PHYSIOLOGY OF INSECT DIAPAUSE. V. ASSAY OF THE GROWTH AND DIFFERENTIATION HORMONE OF LEPIDOPTERA BY THE METHOD OF TISSUE CULTURE

EDMOND L. SCHMIDT [1] AND CARROLL M. WILLIAMS [2]

The Biological Laboratories, Harvard University, Cambridge 38, Mass.

The metamorphosis of insects, like the growth and maturation of vertebrates, is under the control of an endocrine *system.* In the Lepidoptera, the Diptera, the Hemiptera, and the Blattoidea, this system is known to include a minimum of three endocrine tissues—the brain, the prothoracic glands, and the corpora allata. The *brain hormone* is the secretory product of certain highly specialized neurones in the cerebral ganglion; its principal target appears to be the prothoracic glands. Under the tropic stimulation of the brain hormone the prothoracic glands secrete the *growth and differentiation hormone.* And it is apparently this prothoracic gland hormone which reacts with the tissues to promote growth, molting, and metamorphosis (Williams, 1952a). Within the immature insect, there is yet a third hormone whose source is the corpora allata. The corpus allatum hormone is a conservative factor—a *status quo hormone*—which inhibits metamorphosis by modifying the response of the immature tissues to the growth and differentiation hormone (Williams, 1952b).

Though all these conclusions are based on substantial evidence, it is worth recalling that neither the brain hormone, nor the growth and differentiation hormone, nor the *status quo* hormone has ever been demonstrated by any method of chemical or *in vitro* assay. Consequently, it has been necessary to judge the hormonal action by recourse to such indirect methods as the extirpation and implantation of living endocrine organs, parabiosis, and similar procedures. Indeed, none of the above-mentioned hormonal effects is duplicated when one removes blood from an insect and injects it into a test animal. This fact is scarcely mentioned in the literature (Schürfeld, 1935; Plagge, 1938), though it is a reasonable presumption that such experiments have been carried out by most investigators. In studies of the Cecropia silkworm, we have failed in repeated attempts to demonstrate endocrine activity by the injection of blood. Thus, in numerous experiments performed on brainless diapausing pupae, two-thirds of the blood was drained and replaced by that of mature larvae or developing adults. We have continued this procedure on individual pupae at weekly intervals from one to seven times. In no instance did the transfused blood have any detectable effects on the test animal.

[1] Former Predoctoral Fellow of the Lalor Foundation. Present address: Department of Zoology, University of Connecticut, Storrs, Connecticut.

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Experiments of this type have been the principal basis for the view that the hormones of insects are conveyed in the tissues rather than in the blood. If the blood has no demonstrable endocrine activity, then perhaps the hormones of insects, like the auxins of plants (Thimann, 1952), may be transported from cell to cell rather than in the "sap."

With the exception of the blood-borne factor which promotes puparium formation in the higher Diptera (Fraenkel, 1935), the only substantial evidence that hormones concerned with growth and metamorphosis are present in the blood has hitherto been derived from parabiosis experiments. Thus, when two insects are grafted together so that they share the same blood, the one can supply hormones to the other. However, under further analysis, the two animals are found to grow together and attain organic continuity before the hormonal effect is manifested (Bodenstein, 1938; Williams, 1946). Moreover, this tissue union is established even when glass or plastic tubes have been interposed between the animals (Williams, 1947; Lüscher, 1948). The parabiosis experiments have therefore intensified rather than resolved the problem of the mode of transport of insect hormones.

The central and persistent difficulty has been the lack of any practical method of assaying the insect hormones. In the absence of such tests, it has been impossible to detect the hormones in the blood or in the tissues. It has also been impossible to proceed with their extraction, purification, and chemical characterization.

The present study was directed toward the development of an assay for the growth and differentiation hormone. For reasons considered above, it was obvious that the intact insect failed to provide a basis for such a test. Attention was therefore focussed on the culture of an individual tissue in the hope that an *in vitro* system might furnish a method of biological assay.

Practical considerations directed the choice of an appropriate tissue. The latter should be readily available and in a uniform state at the outset. It should undergo prompt response at the beginning of adult development; *i.e.,* during the period when the action of the growth and differentiation hormone is uncomplicated by the simultaneous presence of the *status quo* hormone. Moreover, its response to the growth and differentiation hormone should be accompanied by conspicuous change. Finally, the tissue should be simple to culture.

One tissue above all others appeared to satisfy these requirements; namely, the male sex cells of the diapausing pupal testes—a cell type which at the outset of adult development undergoes prompt and spectacular transformation into spermatozoa. Could such cells be cultured *in vitro?* Assistance in so doing was sought in a survey of the scattered literature.

In Vitro Culture of Insect Tissues

The first attempt to culture insect tissues was that of Goldschmidt (1915, 1916, 1917). Working in Harrison's laboratory, Goldschmidt cultured the male sex cells of Cecropia pupae and observed definite maturation in simple hanging-drop preparations. Unfortunately, no record was made of the exact status of the "pupae" that served as donors of blood; consequently, no endocrinological significance was apparent in Goldschmidt's results.

Similar hanging-drop preparations have subsequently been used to observe meiosis in the male germ cells of adult Orthoptera and Hemiptera (Lewis and

Robertson, 1916; Bêlâr, 1929; Baumgartner and Payne, 1930; Ris, 1949). But in all these instances the germ cells were observed for only a few hours after their isolation, and the development *in vitro* was clearly a continuation of processes already underway in the intact insect.

The only direct support for the view that the *in vitro* growth of insect tissues may depend on hormonal factors is an isolated study reported twenty-five years ago. In experiments designed to solve a morphological problem, Frew (1928) observed that the leg discs of blowfly larvae evaginated and underwent definite development in a culture medium prepared from early pupae, but failed to do so in a medium prepared from older pupae or from larvae. However, Frew's experiments are difficult to interpret since the data are presented in meager detail and technical difficulties are emphasized.

Numerous observations have been made of the behavior of cultured blood cells. In one of the early studies in this field, Glaser (1917) found that the blood cells of caterpillars and adult grasshoppers underwent limited multiplication in simple cultures, either in blood or in Locke's solution. However, several other investigators have been unable to detect mitotic activity in hanging-drop preparations of blood. Goldschmidt (1916) observed only amitotic activity as the blood cells of Cecropia formed a syncytial tissue in the cultures. Similar *in vitro* formations of blood cell syncytia have been observed in *Oryctes* (Lazarenko, 1925), *Periplaneta* (Taylor, 1935; Trager, 1935), larval *Bombyx mori,* and pupal *Platysamia cecropia* (Trager, 1935). Fischer and Gottschewski (1939) report the multiplication of *Drosophila* blood cells in an artificial medium which is not described in detail; growth was also observed in isolated leg and wing anlagen.

But the continuous multiplication of cells, comparable to that obtained in cultures of vertebrate tissues, has apparently not been achieved on insect material. The nearest approach was Trager's (1935) finding that certain cells in the ovary of mature silkworm larvae underwent considerable proliferation when cultured in a medium containing a small quantity of larval blood. Moreover, Charin (1930) reports the migration and limited multiplication of certain lepidopterous tissues *in vitro*—a finding reminiscent of the so-called "residual growth" of vertebrate tissues in the absence of embryonic extract. In the remaining instances in which insect tissues have been successfully explanted (Murray, 1926; Gottschewski and Fischer, 1939; Stern, 1940), residual activity was apparent, but no actual growth occurred.

From this survey of the literature, it is apparent that the cultivation of insect tissues has not been a widely used or highly successful technique. In view of the fact that morphogenetic activity in the insect itself is intermittent and evidently related to growth-controlling hormones, the scanty success achieved in the culture of insect tissues suggests that these same endocrine factors may be required for the growth of isolated tissues.

Development of Sex Cells in the Intact Insect

The testes of the Cecropia silkworm, at all stages, are divided internally into four tubules by three septa radiating from the region of the vas deferens. The tubules contain the germ cells, the majority of which are gathered into small balls and enclosed by an envelope of flattened cells—the whole forming the so-called "germinal cysts" (Dederer, 1907; Omura, 1936). The latter are either free in the fluid which

fills the testicular tubules or suspended in grape-like clusters from minute tracheoles. The maturity of the germ cells can be judged by their size, by the number in each cyst, and by the fact that their early development transforms the cysts into hollow spheres.

During the prepupal period the cells in a few of the more mature cysts undergo one or both maturation divisions, all of the cells in a given cyst dividing simultaneously. In a small proportion of cysts, spermatids begin to differentiate at this time—a process invariably accompanied by considerable elongation of the cyst as a whole. Cells which undergo this precocious maturation during the prepupal period appear to die shortly thereafter. Thus the testes of diapausing pupae include cysts containing degenerate cells with pycnotic nuclei, but no recognizeable spermatids. Consequently, the germ cells in the pupal testes closely resemble those present in mature larvae and range from spermatogonia to primary spermatocytes.

During the pupal diapause there is no indication of further development in any of these cells. However, immediately after the termination of diapause and the initiation of adult development, a rapid and impressive maturation takes place. Mitotic divisions occur in the immature cysts containing spermatogonia, while meiotic divisions occur in the more mature cysts containing primary spermatocytes. These changes are synchronized with the first signs of adult development in the pupal hypodermis. Shortly thereafter, even the immature germ cells, comparable to those commonly observed in larvae midway the fifth instar, promptly transform into spermatids. By the end of the first week of adult development, nearly all the sex cells in the testes have been converted to spermatids.

Materials and Methods

Sex cells from the testes of diapausing pupae of *Platysamia cecropia* and *Samia walkeri* (*cynthia*) were cultured in the blood of larvae, diapausing pupae, developing adults, and adults of the same species. The cultures were prepared as follows:

A male diapausing pupa was selected and surface-sterilized by immersion in a solution of 0.05% mercuric chloride in 50% ethyl alcohol, followed by thorough rinsing in sterile distilled water—a treatment that had no detectable effects on the insects at any stage in development. A transverse slit was then cut in the dorsal region between the fourth and fifth abdominal segments and, with slight pressure on the abdomen, the testes were caused to herniate. Each testis was then transferred to a depression slide containing blood previously collected from a suitable donor.

Rupture of the testicular envelope liberated thousands of germinal cysts into the small pool of blood. By means of a fine pipette a drop of the resulting suspension was placed on the underside of a coverslip and the latter sealed to a depression slide with melted paraffin. It was impossible to pipette a uniform suspension of cysts, and the number included in each culture varied considerably. Ordinarily, each preparation contained at least 75 cysts—indeed, a relatively large number seemed to favor the survival of the culture. Five or six cultures were usually prepared from each sample of blood. Each culture was immediately examined under the compound microscope for any indication of development; it was then placed at the constant temperature of 25° C. or 30° C. and examined at daily intervals.

The blood used as the culture medium was obtained by slicing the cuticle and underlying hypodermis from the facial region of donor insects and draining the

blood into a depression slide containing a few small crystals of phenylthiourea (twice recrystallized from the Eastman product). The blood of these silkworms, like that of most insects, undergoes rapid darkening unless the activity of tyrosinase is inhibited. This darkening results from the enzymatic oxidation of phenols to quinones. Such darkened blood is wholly unsuitable as a culture medium and, in fact, is poisonous. Phenylthiourea specifically inhibits tyrosinase and opposes these changes without, in itself, being toxic. It was the use of this chemical that made the present experiments technically possible.

Blood from developing adults contains a large amount of cellular debris from disintegrating fat body. This was largely eliminated by allowing the particulate matter to settle and withdrawing the relatively clear blood with a pipette. In a number of experiments, the blood was centrifuged to remove all cells—a procedure which seemed to be inconsequential.

The preparation of cultures was accomplished with sterile instruments and glassware; the technique was simple and sufficiently rapid to make elaborate precautions against air-borne organisms unnecessary. Approximately 2% of the cultures eventually showed contamination with microorganisms. Since a number of identical cultures were always prepared, contamination was not a major source of difficulty.

Pupal Germ Cells Cultured in Blood of Pupae, Developing Adults, and Adults

The results of 179 successful cultures prepared in the blood of post-larval donors are summarized in the lower portion of Table I. This total includes all cultures in which any development was detected, though in some instances the cultures survived a relatively short time. Since the onset of development was occasionally delayed until the fourth day, the absence of development was not considered significant until the fourth day; cultures showing no development but failing to survive four days are not included in the data. The death of germ cells *in vitro* seemed to be closely followed or even preceded by visible degenerative changes, and it has been assumed that the cells were alive until these changes appeared.

1. In blood of diapausing and previously chilled pupae

As indicated in Table I, germ cells in the majority of cultures prepared in the blood of pupae which had been in the dormant state for 5 to 16 weeks retained their original configuration (Fig. 1A) and showed no development whatsoever, notwithstanding the fact that many of the cultures survived for a week or longer. In certain preparations the meiotic divisions were evident in an occasional cyst (Fig. 1B). However, the cells in such cysts underwent degeneration within two or three days without forming elongated spermatids, and development was initiated in no additional cysts. On rare occasions, cysts like those shown in Figures 1C and 1D were detected in cultures several days old. Cultures showing any of these patterns of minimal response have been listed as undergoing "slight development."

Results identical to those obtained in the blood of dormant pupae were observed in cultures prepared in the blood of previously chilled pupae—that is, pupae which had been stored at 5° C. for a sufficient period to assure the initiation of development after about two weeks' exposure to 25° C. Altogether it is evident that germ

TABLE I

Pupal germ cells cultured in blood from larvae, prepupae, pupae, and developing adults

Source of blood	Age of donors (days)	No. of donors	No. of cultures	Development in cultures		
				None	Slight	Progressive
Mature 5th instar larvae, not yet spinning	0	3	15	14	1	0
Larvae, spinning outer capsule of cocoon	0–1	3	10	8	2*	0
Larvae, spinning inner capsule of cocoon	1–2	5	34	5	0	29
Larvae, finished spinning cocoon	2–5	3	9	0	3*	6
Green prepupae	5–8	3	11	0	1*	10
White prepupae	8–9	2	10	0	4*	6
Fresh pupae	9–10	2	13	0	2*	11
Diapausing pupae	After 3 weeks at 25° C.	4	17	2	9	6
Diapausing pupae	After 5 to 16 weeks at 25° C.	13	40	26	14**	0
Chilled diapausing pupae	After 12 to 35 weeks at 5° C.	7	23	20	3*	0
Developing adults	1st to 5th day	15	47	0	6*	41
Developing adults	7th to 18th day	6	18	0	2*	16

* All cultures died within 48 hours after development was initiated.
** Six cultures died within 48 hours after development was initiated.

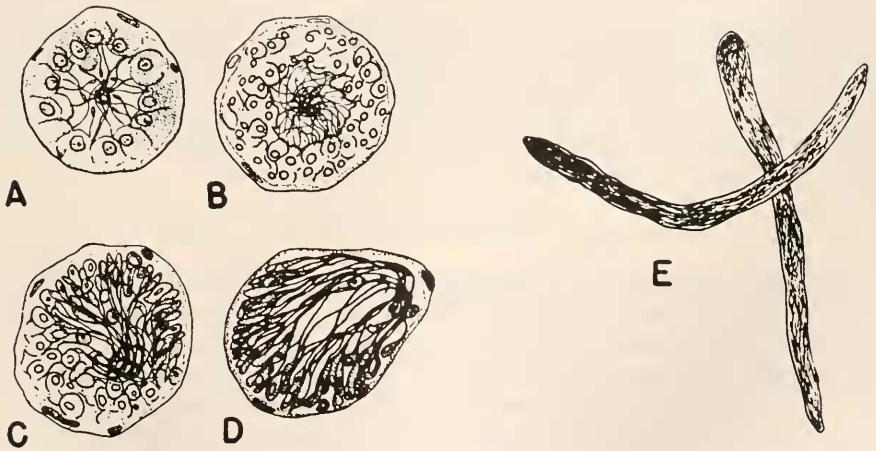

FIGURE 1. Characteristic stages in the development of pupal Cecropia sex cells in hanging-drop cultures of active blood. *A* is an optical section of a testicular cyst from a diapausing pupa; the enclosed cells are primary spermatocytes. *B* shows a typical cyst after several days of culture; the sex cells have just completed meiosis. Development proceeds in *C* to yield early spermatids within the still spherical cyst. In *D* the differentiation of spermatids has continued and the cyst as a whole has begun to elongate. *E* records the final stage of *in vitro* differentiation; the cysts are greatly elongated and contain well-differentiated spermatids. *A* through *D*, × 250; *E*, × 140.

cells are incapable of undergoing rapid or extensive development in the blood of diapausing or previously chilled pupae.

2. In blood of developing adults and adults

With the termination of pupal diapause and the initiation of adult development, a remarkable change was evident in the blood's capacity to promote the development of sex cells. Within 24 to 48 hours after cultures were prepared in the blood of developing adults, the cells in one or more cysts ordinarily showed the completion of the maturation divisions. Within the following 24 hours, meiosis usually occurred in many additional cysts. Meanwhile, the more precocious cysts underwent elongation, the nuclei moving to one pole and the axial filaments of the developing spermatids growing towards the other. At this stage a large proportion of the cysts corresponded to those diagrammed in Figures 1B to 1D. After a week in culture, the majority of cysts showed definite elongation; many were ten to fifteen times longer than wide and contained well differentiated spermatids (Fig. 1E).

Since the germ cells usually failed to survive longer than a week in culture and not infrequently died sooner, the final degree of differentiation was less impressive in those cultures where development was delayed or proceeded slowly. Characteristically, however, continuous progress was made toward the spermatid stage as long as the germ cells survived. Accordingly, "progressive development" was distinguished, not so much by the absolute amount of differentiation, as by its sustained progress. The results in Table I show that progressive development occurred in blood obtained from animals after the initiation of adult development.

3. Germ cells of Platysamia cecropia cultured in the blood of Samia walkeri

Cultures prepared from blood and testicular tissues of *Samia walkeri* showed the same behavior as those prepared from *Platysamia cecropia*. Moreover, the sex cells of *Platysamia* developed as promptly in the post-pupal blood of *Samia* as in the post-pupal blood of *Platysamia* itself. Progressive development also occurred in experiments of the reverse type; that is, when the germ cells of *Samia* pupae were cultured in blood obtained from developing adults of *Platysamia*.

Blood Cells and Spermatogonia In Vitro

In cultures prepared in uncentrifuged blood, many of the blood cells flattened themselves against the coverslip and within two days coalesced to form small areas of syncytial tissue. Little further change occurred when the blood cells of diapausing pupae were cultured in the blood of diapausing pupae, though the cells appeared to survive for over a month. In contrast, in cultures prepared in the blood of developing adults, a slow increase in the size and complexity of the blood cell syncytia became evident after five or six days. In cultures maintained for two weeks or longer and subsequently fixed and stained, scattered mitotic figures were evident in the flattened blood cells.

In cultures prepared in the blood of early developing adults and maintained for longer than the usual period of one week, masses of small cells slowly appeared. These cells failed to correspond to any of the various types of blood cells, nor did

they show the latter's tendency to flatten and form syncytial tissues. Inspection of fixed and stained cultures revealed frequent mitotic figures.

Pupal testes invariably contain a small number of cells not yet enclosed within cysts. These cells, which are presumed to correspond to early spermatogonia, are inevitably released along with the cysts of late spermatogonia and spermatocytes when the testes are ruptured in the preparation of the cultures. The cells observed to multiply in cultures closely resembled the early spermatogonial cells in terms of the presence of a scanty, dense cytoplasm and a large vesicular nucleus.

It thus appears that the property of the blood of developing adults which promotes the differentiation of spermatocytes also stimulates cells of a different character, such as blood cells and spermatogonia, to divide. Consequently, it can truly be said that the active agent in the blood of developing adults promotes both growth and differentiation.

Pupal Sex Cells Cultured in Larval Blood

Observations were made of the differentiation of pupal cells when cultured in larval blood. The preparation of such cultures was at first complicated by a jelly-like clotting of the blood when withdrawn from caterpillars. Fortunately, this viscous change, due apparently to adhesions between blood cells, disappeared when the blood was stored for 12 to 24 hours at 3° C. Under this circumstance, the cells formed dense clumps and the clear fluid residue could once more be used in culturing the pupal spermatocytes.

The results of cultures of pupal cysts in larval blood (Table I) indicate that the blood of mature fifth instar larvae is essentially inactive until a stage signalled by the spinning of the inner coat of the cocoon. The larval blood then becomes active in promoting the transformation of pupal sex cells. From these results, it appears that a factor required for the maturation of pupal cells increases markedly in concentration, not only at the outset of adult development, but also just prior to pupation. This factor, as indicated in Table I, persists in the blood throughout the prepupal period and then slowly falls to sub-threshold concentration following pupation.

Sex Cells Cultured in Diluted Blood and in Mixtures of Active and Inactive Blood

The failure of the blood of diapausing pupae to support the maturation of sex cells might be attributed either to the presence of an inhibitory factor, or to the absence of a necessary growth hormone. To distinguish between these possibilities, cultures were prepared in mixtures of "dormant" blood and "active" blood and in blood diluted with insect Ringer's solution (Ephrussi and Beadle, 1936).

As recorded in Table II, the low activity of dormant blood was not altered by the addition of an equal volume of Ringer's solution. Hence, if the diapausing blood contains an inhibitory substance, the latter is not rendered sub-threshold by this degree of dilution. Table II also shows that active blood remained active after the addition of an equal volume of dormant blood. These findings indicate that the blood of developing adults contains a growth-promoting hormone which is in far lower titer in the blood of mature diapausing pupae.

TABLE II

Pupal germ cells cultured in mixtures of bloods and in bloods diluted with Ringer's solution

Culture medium	Number of experiments	Number of cultures	Development in cultures		
			None	Slight	Progressive
Blood from dormant pupae plus Ringer's solution; equal parts	4	12	8	4*	0
Blood from developing adults plus Ringer's solution; equal parts	5	17	0	0	17
Blood from developing adults and dormant pupae; equal parts	5	22	0	12**	10

* All cultures died within 48 hours after development was initiated.
** Seven cultures died within 48 hours.

PROPERTIES OF THE GROWTH-PROMOTING SUBSTANCE PRESENT IN ACTIVE BLOOD

The nature and properties of the growth-promoting hormone in the blood of pupating larvae and developing adults were studied by subjecting active blood to various treatments and then re-testing its capacity to promote development *in vitro*.

1. Effects of storage at 3° C. and of freezing

Hormonal activity was retained when active blood was stored under refrigeration. As previously mentioned, blood collected from pupating larvae was routinely stored at 3° C. for 12 to 24 hours without loss of activity. Indeed, in two experiments involving a total of eight cultures, progressive development was observed in prepupal blood previously stored at 3° C. for four days. In another experiment, blood obtained from developing adults was frozen at − 29° C., then thawed at room temperature, then re-frozen and re-thawed. When tested in six cultures, full activity was observed—a finding which demonstrates that the hormone is stable at low temperatures.

2. Effects of heat treatment

Abrupt heating to temperatures above 60° C. caused the blood to coagulate in a solid mass. This difficulty was avoided by raising the temperature in steps of approximately 5°, and removing the precipitate by centrifugation after each step. By this procedure a small fraction of clear supernatant could be obtained even after heating the blood to 100° C.

When active blood was heated to 56° C., a scanty white precipitate formed; after centrifugation the supernatant proved suitable for use in the cultures. But when the temperature was raised to 60° C., a copious white precipitate appeared; the supernatant, when tested, now caused extreme fragmentation and granulation of the sex cells and death within a few hours—a result which we attribute to the altered osmotic pressure of the blood. Consequently, in order to test blood previously heated to temperatures above 60° C., it was necessary to mix the supernatant with an equal volume of untreated, inactive blood obtained from diapausing pupae.

When prepared in this manner, the culture medium regained its compatibility with the germ cells. In each such experiment the inactivity of the unheated diapausing blood was verified in control cultures.

Experiments testing the growth of spermatocytes in initially active blood are summarized in Table III. Pupal germ cells showed prompt and progressive development to spermatids when cultured in blood previously heated for fifteen minutes at 75° C. or at any lower temperature. In contrast, the behavior of cultures prepared with blood heated for ten minutes at 80° C. was indicative of a definite decrease in growth-promoting activity. Though three of four such cultures showed slight development after three days, no further development occurred, notwithstanding the fact that the cells survived for approximately seven days. The fourth culture likewise appeared to survive for about a week, but showed no development whatsoever. A similar negative result was observed in blood previously heated to 100° C. for ten minutes, though in three cultures the cells survived for at least six days. In

TABLE III

Pupal germ cells cultured in media containing active blood previously heated to various temperatures

Preliminary heat treatment		Number of satisfactory cultures	Development in cultures
Temp. (° C.)	Time (min.)		
56	25	10	Progressive
65*	15	7	Progressive
70*	15	6	Progressive
75*	15	5	Progressive
80*	10	4	Slight
100*	10	3	None

* Supernatant diluted with equal parts of inactive blood.

summary, these results indicate that the blood's hormone is stable and unprecipitated by brief exposure to a temperature of 75° C., but is progressively destroyed or precipitated at higher temperatures.

3. *Effect of dialysis*

Approximately one ml. of active blood was placed in a viscous casing and dialyzed for 24 hours at 1° C. against 100 ml. of insect Ringer's solution. The undialyzed fraction, mixed with an equal volume of blood from dormant pupae, was tested in five cultures and found to retain its capacity to promote the development of pupal germ cells. The experiment was repeated, increasing the time of dialysis to two days. The undialyzed fraction, mixed with an equal volume of dormant blood, caused prompt development when tested. Evidently, the hormone is either a large molecule or is closely bound to such a non-dialyzable component.

DISCUSSION

The tissue culture studies demonstrate that the blood of the Cecropia silkworm contains a growth factor whose concentration or activity undergoes systematic

change during the larval-pupal-adult transformation. Its initial low titer in the blood of the mature larva increases markedly just prior to the prepupal stage; *i.e.,* during the period when the growth and differentiation hormone induces the larva to pupate (Williams, 1952a). Within the newly formed pupa the factor then persists in gradually diminishing titer, only a trace being encountered in the blood of the diapausing pupa several weeks later.

For months thereafter the blood of the diapausing pupa remains virtually inactive. But just prior to the termination of diapause and during the ensuing first week of adult development the factor reappears in high titer—a change which correlates precisely with the period of action of the prothoracic glands (Williams, 1952a). For these several reasons we are persuaded that the factor which promotes the *in vitro* development of the sex cells is the same growth and differentiation hormone which, within the intact insect, is required for the growth and maturation of the gonads and of all other tissues (Williams, 1952b).

Efforts were made to demonstrate this fact by more direct procedures; namely, by the implantation of active prothoracic glands into cultures prepared in inactive blood. Experiments of this type were complicated by bacterial contamination of the fragments of the prothoracic glands during their dissection from developing adults. Eventually, three hanging-drop cultures were prepared containing both germinal cysts and fragments of living prothoracic glands. Though the sex cells and the glandular cells appeared to survive for several days, no development took place. Similar negative results were obtained in cultures fortified with extracts of active prothoracic glands in saline or in inactive blood.

The apparent failure of the prothoracic glands to continue their secretory activity under the highly artificial conditions existing in hanging-drop cultures is not surprising, particularly in view of Fukuda's (1944) finding that a normal oxygen supply is necessary for the secretory activity of the prothoracic glands—a condition obviously not met in small, sealed cultures.

If one accepts the tissue culture test as a valid assay for the growth and differentiation hormone, then it is clear that the isolated testicular tissue is far more sensitive to the hormone than are the intact testes or the insect as a whole. The isolated germ cells respond to concentrations of growth and differentiation hormone that are sub-threshold within the intact insect. Thus the sex cells developed promptly when cultured in the blood of recently pupated animals in which all development had ceased (Table I). Indeed, the blood of pupae which had been dormant for months not infrequently caused a slight but definite response of the cultured cells. Moreover, it is worth recalling that during the second week of adult development the blood showed considerable hormonal activity, notwithstanding the fact that the prothoracic glands, the source of the hormone, are known to undergo complete degeneration during the first week of adult development. While attesting to the stability of the growth and differentiation hormone within the insect, this finding suggests the further possibility that the hormone may continue to influence the development of the insect during periods when the prothoracic glands are inactive or even absent, as, for example, during the maturation of the eggs.

There is clear evidence that one reason for the high sensitivity of the *in vitro* procedure is the absence of the testicular envelopes which are interposed between the sex cells and the blood within the intact insect. During the period of prepupal

development this barrier apparently shields the germ cells from the threshold concentration of hormone in the surrounding blood. For if the sex cells are removed from the testes of prepupae and cultured in the blood of the very same animals, normal spermatogenesis is observed. And, as we have seen, the same is true within the newly formed pupa where the concentration of hormone is sufficient to cause spermatogenesis *in vitro* but not *in vivo*.

The sensitive response of cultured germ cells affords the first direct evidence that the growth and differentiation hormone of insects is, indeed, a blood-borne hormone; it also provides a means of recognizing this factor. The fact that the germ cells of either *Platysamia cecropia* or *Samia walkeri* develop in the blood of the opposite species demonstrates that the growth and differentiation hormone of Lepidoptera is, to this degree, neither species- nor genus-specific. The hormone also appears to be non-dialyzable and destroyed or precipitated by exposure to temperatures above 75° C.—properties which suggest that the growth and differentiation hormone is either a protein or a smaller molecule tightly conjugated to a protein. Though the tissue culture technique is only roughly quantitative in its present form, the growth and differentiation hormone, according to the evidence already at hand, appears to be relatively stable within the insect and to wax and wane in synchrony with the recurrent bouts of secretory activity by the prothoracic glands.

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Summary

1. Spermatogonia and spermatocytes isolated from the testes of dormant pupae of the silkworms, *Platysamia cecropia* and *Samia walkeri* (*cynthia*), promptly develop into well differentiated spermatids when cultured in hanging-drops of blood obtained from pupating larvae or developing adults of these species. Comparable development does not occur in cultures prepared in blood obtained from diapausing pupae or from mature larvae prior to a well-defined period before the onset of the prepupal stage.

2. The *in vitro* development of the sex cells signals the presence of a growth-promoting hormone. In inactive blood, as during the pupal diapause, this hormone is ordinarily in sub-threshold titer.

3. In addition to promoting the maturation of spermatocytes, the hormone causes the *in vitro* multiplication of blood cells and spermatogonia.

4. Pupal blood is active in promoting the development of germ cells *in vitro* after the initiation of adult development; the blood of mature larvae becomes active at a stage signalled by the spinning of the inner coat of the cocoon. This timing corresponds precisely with the periods during which the prothoracic glands secrete the growth and differentiation hormone which provokes the pupation of the larvae and the adult development of the pupa.

5. Evidence is presented that the development of the germ cells *in vitro* reflects the presence in the culture medium of the growth and differentiation hormone secreted by the prothoracic glands. The development of the sex cells in the tissue culture appears to be an exceptionally sensitive test for this hormone.

6. By the use of this test the hormone was found to be non-dialyzable and destroyed or precipitated by exposure to temperatures higher than 75° C. Accordingly, it is concluded that the growth and differentiation hormone is either a protein or a smaller molecule conjugated to a protein.

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